

**Amendments to the Specification:**

- On page 1, line 1, please replace the Title with the following Title:

MODULATORS OF SMURF AND ANTAGONISTS OF BMP AND TGFB  
BMP/TGF $\beta$  SIGNALING PATHWAYS

- On page 5, please replace the paragraphs spanning lines 18-26 with the following paragraph:

**Figure 1 Figures 1A-D.** *Smurf1* encodes an E3 ubiquitin ligase. Protein sequence of Xenopus and human Smurf1 compared to yeast (*S. pombe*) pub1, given as SMURF1, hSMURF1 and PUB1, respectively in the figure. Identical amino acids are shaded dark gray and conservative substitutions are shaded light gray. Based on primary structure, Smurf1 and pub1 are members of the Hect family of E3 ubiquitin ligases and display several conserved features of the family: A lipid/Ca<sup>2+</sup> binding domain is located at the N-terminus (residues 22-37), two WW protein interaction domains at 236-271 and 282-311 (indicated by thick lines), and lines), and a catalytic Hect domain beginning at residue 347 and extending to the C-terminus. Alignment was by Clustal-W CLUSTALW analysis (MacVector).

- On page 7, please replace the two (2) paragraphs spanning lines 7-16 with the following two paragraphs:

**Figures 5A, 5B, and 5C, and 5D.** *hSmurf1* regulates *Smad1* turnover and ubiquitination: *hSmurf1* enhances *Smad1* turnover.

**(A) and (D)** COS-1 cells were transiently transfected with pCMV5-Smad1 alone or with ~~Flag-tagged~~ FLAG-tagged hSmurf1 (F/hSmurf1) using ~~lipofectAMINE~~ LIPOFECTAMINE. Two days later, transfectants were subjected to pulse-chase analysis using [<sup>35</sup>S]methionine. At the indicated time during the chase, cells were lysed and subjected to an  $\alpha$ -Smad1 immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography (~~left panel~~) (Fig. 5A). Radiolabelled Smad1 was also quantitated by phosphoimaging, and the results are plotted as the amount of [<sup>35</sup>S]

methionine-labelled Smad1 present at each time point relative to the level at time 0 (~~right panel~~) (Fig. 5D).

- On page 9, please replace the three (3) paragraphs spanning lines 33-35 with the following three paragraphs.

**Figure 9. Figures 9A-F. cDNA sequence of human Smurf1 [SEQ ID NO:1].**

**Figure 10. Figures 10A-C. Protein sequence of human Smurf1 [SEQ ID NO:2].**

**Figure 11. Figures 11A-H. cDNA sequence of human Smurf2 [SEQ ID NO:3].**

- Please replace the paragraph spanning page 11, line 32 - page 12, line 2 with the following paragraph:

(C) *Smurf2 increases the turnover rate of the receptor complex.* COS-1 cells transfected with ~~TGF $\beta$  receptors~~ TGF $\beta$  receptors (T $\beta$ RII-HA and T $\beta$ RI-HA) alone or together with Smad7-HA, ~~Flag-Smurf2~~ FLAG-Smurf2 or both were pulse-labelled with [<sup>35</sup>S]-methionine and then chased for the indicated times in media containing unlabelled methionine. Cell lysates were subjected to anti-HA immunoprecipitation and the amount of labelled receptors and Smad7 was quantified by phosphorimaging and is plotted relative to the amount present at time 0 (Figs. C1-4).

- Please replace the paragraph spanning page 21, line 33 - page 22, line 4 with the following paragraph:

Similarly, in a particular embodiment, two amino acid sequence are “substantially homologous” or “substantially similar” when greater than 70% of the amino acids are identical, or greater than about 90% are similar (functionally similar). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program, BLAST, and ~~Clustal-W~~ CLUSTALW analysis

(MacVector). Sequence comparison algorithms are known in the art. ~~can also be found at~~  
~~<http://www.nwfsc.noaa.gov/bioinformatics.html>~~

- **On page 47, please replace the paragraph spanning lines 19-27 with the following paragraph:**

*Co-immunoprecipitation.* For immunoprecipitation assays, *Xenopus* Smad1 (36), mouse Smad4, and human Smad2 (47), were FLAG-tagged at their C-termini and translated *in vitro* (rabbit reticulocyte extracts; Promega) in the presence of <sup>35</sup>S-Met. The FLAG-tagged Smads were bound to anti-FLAG antibody-conjugated beads (Kodak), washed in co-IP buffer (10mM ~~Tris~~ TRIS (tris(Hydroxymethyl)aminomethane), pH 7.5, 90 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 10% glycerol, 1 mM ~~phenylmethylsulfonylflouride~~ phenylmethylsulfonylfluoride) then incubated with <sup>35</sup>S-Met-labelled Smurf1 in the same buffer. After washing in co-IP buffer and elution in gel loading buffer, proteins were separated by SDS-PAGE and visualized by autoradiography.

- **Please replace the three (3) paragraphs spanning page 48, line 9 - page 49, line2 with the following three paragraphs:**

*Immunoprecipitation and Immunoblotting.* COS-1 and 293T cells were transiently transfected using ~~lipofectAMINE~~ LIPOFECTAMINE (GibcoBRL) and calcium phosphate precipitation methods, respectively (49). Immunoprecipitations and immunoblotting were performed as described previously (50) using ~~anti-Flag~~ anti-FLAG M2 monoclonal antibody (Sigma), anti-Smad1/5 polyclonal antibody (50) or anti-WW2 Nedd4 (51) polyclonal antibody. Detection was achieved using the appropriate HRP conjugated goat anti-mouse or goat anti-rabbit secondary antibodies and enhanced chemiluminescence (Amersham).

*Pulse Chase Analysis.* COS-1 cells were transfected as indicated above. Two days post-transfection, the cells were labelled for 10 min. at 37° C with 50 µCi [<sup>35</sup>S]-methionine/ml in methionine-free DMEM (Pulse). Cell layers were then washed two times and incubated in DMEM+10% FCS for the indicated time periods (Chase). At each time point of the chase, cell lysates prepared in TNTE lysis buffer (50 mM ~~Tris/HCL~~ TRIS/HCL,

pH 7.4, 150 mM NaCl, 0.5% Triton X-100 and 1 mM EDTA) containing protease and phosphatase inhibitors were subjected to immunoprecipitations using an anti-Smad1 polyclonal antibody. Immune complexes were resolved by SDS-PAGE and visualized by autoradiography. A ~~Phosphorimager~~ phosphorimager (Molecular Dynamics) was used to quantitate the amount of metabolically labelled Smad1 present at each time point.

*Ubiquitination Assay.* 293 T cells were transfected with HA-tagged ubiquitin, untagged-Smad1, and either ~~Flag-hSmurf1 or Flag-hSmurf1~~ FLAG-hSmurf1 or FLAG-hSmurf1 (C710A) as indicated above. Two days post-transfection, cells were lysed and subjected to ~~a Smad1 immunoprecipitation~~ a Smad1 immunoprecipitation. The immunoprecipitates were then washed sequentially two times each in TNTE + 0.1% ~~triton~~ Triton, SDS-RIPA (TNTE lysis buffer, 0.1% sodium dodecyl sulfate and 1% deoxycholate), and 500 mM LiCl, 50 mM ~~Tris-HCL~~ TRIS/HCL, pH 7.4 and 0.1% ~~triton~~ Triton. The presence of HA-ubiquitinated Smad1 in the immune complexes was visualized by SDS-PAGE followed by immunoblotting with the monoclonal anti-HA 12CA5. Protein levels of untagged Smad1, ~~Flag-hSmurf1 and Flag-hSmurf1~~ FLAG-hSmurf1 and FLAG-hSmurf1 (C710A) were analyzed by immunoblotting aliquots of total cell lysates with the appropriate antibodies.

- **On page 51, please replace the paragraph spanning lines 24-33 with the following paragraph:**

To investigate whether Smurf1 activity is exclusive to Smad1, we investigated its effects on Smad2, a receptor regulated Smad that functions in TGF $\beta$  and activin signaling pathways. Unlike Smad1, which was sensitive to the lowest doses of hSmurf1, there was only a slight effect on steady-state levels of Smad2 protein, which occurred only at the highest levels of ~~Flag-hSmurf1~~ FLAG-hSmurf1 expression (**Fig. 4C**). Other Smads were tested: ~~Flag-hSmurf1~~ and FLAG-hSmurf1 had little or no effect on Smad3 or Smad4 protein levels, but it elicited a strong decrease in Smad5 protein, which is closely related to Smad 1 (**Fig. 4D**). Together these data demonstrate that hSmurf1 preferentially regulates the steady-state levels of Smad1 and Smad5, two receptor-regulated Smads that function in BMP signaling.

- On page 52, please replace the paragraph spanning lines 9-23 with the following paragraph:

To determine the mechanism of hSmurf1-mediated turnover of Smad1, Smad1 ubiquitination in intact cells was assessed. To facilitate detection of ubiquitin, 293T cells were transfected with HA-tagged ubiquitin together with Smad1, in the presence or absence of ~~Flag-Smurfl~~ FLAG-Smurfl-1. In the absence of hSmurf1, Smad1 displayed little or no detectable ubiquitination. However, upon co-transfection with hSmurf1 we observed the appearance of a ladder of ubiquitin-conjugated Smad1 (**Fig. 5B**). To confirm that ubiquitination of Smad1 required the catalytic activity of the Hect domain in hSmurf1, a point mutant in hSmurf1 (hSmurf1 (C710A)) was constructed. This residue is critical for the catalytic activity of the Hect domain and the mutation is thought to target the cysteine residue that forms a thiolester bond with ubiquitin (22). In contrast to wildtype hSmurf1, expression of hSmurf1 (C710A) did not yield ubiquitinated Smad1. Moreover, hSmurf1 (C710A) did not affect Smad1 steady-state protein levels compared to wildtype hSmurf1, despite efficient expression of the mutant protein (**Fig. 5C**). Together, these data suggest that hSmurf1 alters Smad steady-state levels by inducing ubiquitin-mediated degradation of ~~Smad1~~ Smad1 ~~via~~ its Hect domain.

- On page 53, please replace the paragraph spanning lines 1-12 with the following paragraph:

To test the capacity of <sup>35</sup>S-labelled Smurf1 to bind and immunoprecipitate Smads *in vitro*, and to test the specificity of Smurf1-Smad interactions in intact cells, the association of hSmurf1 with various Smads in 293T cells was investigated. Wild type hSmurf1 did not detect interactions with Smad1. However, it is possible that in intact cells Smurf1-Smad1 interactions are transient in nature, since demonstrating association between ubiquitin ligases and their substrates has proven difficult in other systems. By examining Smad1 interaction with the ubiquitin-ligase mutant ~~Flag-hSmurf1~~ FLAG-hSmurf1 (C710A),

association of the proteins could be detected (**Figs. 6B and 6C**). Furthermore, this interaction was unaffected by coexpression of the constitutively active BMP-type I receptor, ALK2 (data not shown), and is consistent with the notion that hSmurf1 regulates Smad1 turnover independent of BMP signaling. hSmurf1 (C710A) also bound efficiently to Smad5, but analysis of association with Smad2 revealed little, if any, interaction (**Fig. 6A and 6B**).

- **Please replace the two (2) paragraphs spanning page 59, line 3 - page 60, line 2 with the following two paragraphs:**

*Construction of Plasmids.* For mammalian expression constructs of Smurf2, the open reading frame was amplified by polymerase chain reaction (PCR) and was subcloned into pCMV5 in frame with an amino-terminal ~~Flag~~ FLAG or Myc tag (69). For Smurf2 WW domain deletions, amino acids 163-185 for DWW1, 257-279 for DWW2, and 303-325 for DWW3 were deleted. To generate the catalytically-inactive ubiquitin-ligase mutant of Smurf2, cysteine 716 was replaced by alanine. To generate the Smad7 PY mutants, tyrosine 211 was replaced with alanine (Y211A) or the PPPPY sequence between amino acid residues 206-212 was deleted ( $\Delta$ PY). For ~~T $\beta$ RI-Flag, a Flag~~ T $\beta$ RI-FLAG, a FLAG tag was introduced at the carboxy terminus of the receptor. All constructs were generated by PCR and confirmed by sequencing. The bacterial expression vectors, pET15-Smad7-HA and pGEX4T-1 Smurf2, were generated using convenient restriction sites.

*Immunoprecipitation, Immunoblotting, and Affinity-labelling.* For studies in mammalian cells, 293T and COS-1 cells were transiently transfected using calcium phosphate precipitation, or the DEAE-dextran method, respectively. Immunoprecipitation and immunoblotting were carried out using anti-HA monoclonal (12CA5, Boehringer), anti-HA rabbit polyclonal (Santa Cruz), anti Myc monoclonal (9E10 ascites, Developmental Studies Hybridoma Bank), ~~anti-Flag~~ anti-FLAG M2 monoclonal (Sigma) or anti-Smad7 rabbit polyclonal antibodies. For anti-Smad7 antibodies, rabbits were immunized with bacterially-produced GST-Smad7 encoding amino acids 202-260. After absorption of the antibody to either protein G or A-Sepharose, the precipitates were washed five times with TNTE 0.1% (50 mM ~~Tris~~ TRIS, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100), separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the

appropriate antibody. Detection was conducted using the appropriate horseradish peroxidase (HRP)-conjugated sheep anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (Amersham). Bacterially-produced His-Smad7-HA was incubated with either Ni<sup>2+</sup>-NTA beads (Qiagen) or with GST or GST-Smurf2-bound glutathione beads (Amersham), washed three times with TNTE (0.5% Triton X-100) and precipitates were analyzed by immunoblotting with anti-HA antibodies. For affinity-labelling, transfected COS-1 cells were incubated with 250 pM [<sup>125</sup>I]TGF- $\beta$ 1 at 4°C for 1 h, and receptors were cross-linked to ligand with DSS as described (70). The amount of T $\beta$ R1 bound to Smurf2 or Smad7 was quantified by phosphoroimaging (Molecular Dynamics).

- On page 60, please replace the two (2) paragraphs spanning lines 18-31 with the following two paragraphs:

*Subcellular localization by Immunofluorescence Deconvolution Microscopy.* Mv1Lu cells, plated on gelatin-coated ~~Permanox~~ PERMANOX CHAMBER SLIDES (Nunc), were transfected with the indicated constructs by the calcium phosphate precipitation method. The cells were fixed, permeabilized, and reacted with the primary and secondary antibodies as described (69). Images were obtained using the ~~Olympus~~ OLYMPUS 1X70 inverted microscope equipped with fluorescence optics and ~~Deltavision~~ DELTA VISION deconvolution microscopy software (Applied Precision).

*Transcriptional Response Assay.* HepG2 cells were transiently transfected using the calcium phosphate DNA precipitation method with CMV- $\beta$ gal, 3TP-Lux reporter construct and Smad7-HA constructs as indicated. Total DNA was kept constant by the addition of pCMV5 empty vector. The next day, cells were incubated overnight with or without 100 pM TGF $\beta$ . Luciferase activity was measured using the luciferase assay system (Promega) in a ~~Berthold Lumat LB~~ BERTHOLD LUMAT LB 96V luminometer and was normalized to  $\beta$ -galactosidase activity.